REMARKS

The Office Action of June 1, 2001 has been received and carefully noted, and the comments set forth below are a complete response thereto.

Claims 1-12, 15 and 16 are all the pending claims. By this Amendment, claims 3, 5, 6 and 15 are amended as follows:

Claim 3 now recites a method for obtaining a feedstuff additive (Reference Example 1);

Claim 5 now recites an agent comprising the lipopolysaccharide as the active component (page 9, lines 10-12);

Claim 6 is now directed to a agent comprising the lipopolysaccharide admixed with a feed (page 9, lines 10-12); and

Claim 15 is now properly dependent from claim 1 and has been amended into Markush group format.

Claims 7 and 8 have been canceled without prejudice or disclaimer.

No new matter has been added, and consideration and entry of amended claims 3, 5, 6 and 15 is requested.

I. Response to Objection to the Title

Applicants have substituted the original title with the aforementioned title, and have thereby overcome the Examiner's objection.

II. Formal Matters

a. Response to Objection to Claim to Priority

Applicants' have amended the specification to include a priority statement which

meets and overcomes the objection to the claim to priority.

b. Response to Objection to the Specification

Applicants are replacing the original specification with an amended, substitute specification enclosed herewith, along with a marked-up copy of the original. The informalities have been corrected throughout the disclosure which meets and overcomes all of the Examiner's objections.

More specifically, in responding to the Examiner's comment that the phrase, "Pantoea agglomerans-carrying bacteria" is indefinite, Applicants have replaced this expression with "a sample containing bacterial strains of Pantoea agglomerans".

III. Response to Rejection of Claims 3 and 7 under 35 U.S.C. § 101

Claims 3 and 7 are rejected under § 101 for improperly reciting a "use".

In having amended claim 3 to recite a method and in canceling claim 7, Applicants have rendered the Examiner's rejection moot.

IV. Response to Rejection of Claims 1-12, 15 and 16 under 35 U.S.C. § 112, first paragraph.

The Examiner asserts that the presently claimed invention is only enabled for the three species of crustaceans and fish specifically mentioned in the Specification. The Examiner rejects the claims because they encompass all species of crustacea and fish. Additionally, claim 15 recites fifteen different infectious diseases, and the Examiner does not find support in the Specification for the efficacy of these treatments for all fifteen claimed diseases.

Applicants traverse for the following reasons.

The immune mechanism of crustaceans is similar among the lobster, shrimp,

prawn and crab. Furthermore the immune mechanism for fish is similar for all fishes. Applicants submit that the similarities underlying the immune mechanisms for crustacea and fish, respectively, would be well known to one of ordinary skill in the art. Accordingly, for the instant disclosure, more than adequate enablement is provided for crustacea as well as fish. Applicant's direct the Examiner's attention to Examples 1, 3,4 and 5 describing the effects of the LPS on prawn, Example 2 showing the effects of the LPS on black carp, a fresh-water fish, and Examples 6 and 7 showing the effects of the LPS on yellowtail, a salt-water fish.

Additionally, Applicants wish to draw the Examiner's attention to the numerous U.S. patents where the genera of crustaceans and fish are claimed, but where only one or a very small number of species embodiments are described or exemplied. For example, Yabiki (R2, USPN 5,268,357) cited in the instant Office Action, broadly teaches fish at Col. 7, line 18 and in Table 6, yet the term "fish" has been granted in the claims. Takahashi (R5, USPN 5,641,761) also cited in this Office Action, discloses examples of kuruma prawn, yet the term "crustacea" has been granted in the claims.

Other U.S. patents (copies of which are included with the attached IDS) which exemplify this more liberal practice include, for example, Nikl (USPN 5,189,028), which only discloses species embodiments for salmon still yet the phrase "immune system of fish" is considered enabled vis-à-vis its granting in a claim. Finally, Dessen (USPN 5,573,792) includes examples for only salmon fry (Col. 3, line 26 and Tables 2 and 3) yet the phrase "method of growing fish or crustaceans" was found enabled.

Applicants submit that in view of the abundant number of examples in the original specification and the precedence for granting broadly drawn claims to crustacea or fishes in other U.S. patents, that the Examiner's enablement rejection is unduly burdensome on Applicants. Accordingly, withdrawal of the rejection is requested.

V. Response to Rejection of Claims 1-12, 15 and 16 under 35 U.S.C. § 112, second paragraph.

- in response to the examiner's rejection of the term "the perish" in the claims, Applicants have replaced the term with "activating immunity or preventing infection" or canceled the claims, as appropriate;

- in claim 15, "infectious diseases **is**" has been amended to recite "the infection caused by an infectious microbe selected from the group consisting of...". The claim is now properly dependent from claim 1 and now recites the infectious microbes in Markush group format;

- in Claim 15 the term "streptococcic" has been corrected to recite "streptococcal";

- in Claim 1, the term "substantially" may be a relative term, but it is often held to be definite because one skilled in the art would understand from the instant specification (page 7 at lines 3-5) what is being claimed (MPEP §2173.05(b)).

- in claim 15, the term "vivrio diseases" is well known in the art. *Vivrio* cholerae is synonomous with vivrio diseases, and vivrio diseases occur in both crustacea and fishes.

VI. Response to Rejection of Claims 1-12 and 16 under 35 U.S.C. § 102(b)

Claims 1-12 and 16 are rejected under § 102(b) as being anticipated by Soma et al.

The Examiner considers the instant claims *prima facia* anticipated by Soma since according to the Examiner, Soma discloses gram negative bacteria having LPS of molecular weights ranging between 5000 ± 1000 and 6500 ± 2500 as measured by

SDS page method. Soma teaches the products for use as immunostimulatory agents in different animals. Further, the administration of these stimulators is taken orally. Furthermore, Pantoea agglomerans are taught as a strain of bacteria used to produce the lipopolysaccharides.

Applicants traverse for the following reasons.

Soma does not explicitly or implicitly teach adding these lipopolysaccharides to feed, and then subsequently feeding this fortified feed to fish or crustacea. Since Soma fails to teach or suggest all of the instant claimed elements, Soma is an improper basis for rejection under § 102 for any claim reciting these limitations. Accordingly, withdrawal of the rejection is deemed proper.

VII. Response to Rejection of Claims 1-12, 15 and 16 under 35 U.S.C. § 103(a)

Claims 1-12, 15 and 16 are rejected under § 103(a) as being obvious over Yabiki et al. in view of Rorstad and further in view of Soma et al.

The Examiner considers the instant claims *prima facia* obvious over Yabiki, Rorstad and Soma since Soma teaches feed and feed additives for fish and other animals to increase disease resistance, Yabiki teaches combining additives with commercial feed for rainbow trout in the prevention of viral diseases, and Rorstad et al. teaches enhancing the resistance of fish and crustaceans to disease by stimulating their immune systems. The Examiner makes further reference to Soma for teaching the use of Pantoea agglomerans and gram-negative bacteria.

The Examiner alleges that it would have been obvious to replace the high molecular weight molecules of Yabiki and Rorstad with the low molecular weight of lipopolysaccharides of Soma in order to obtain the instant claimed invention.

Applicants traverse for the following reasons.

Rorstad and Yabiki are specifically silent with respect to using low molecular weight polysaccharides, and Applicants submit that the Examiner's rejection of the claims is based on improper hindsight analysis.

The Examiner's attention is further directed to Example 5 of the present specification, more specifically Group 5 which demonstrates comparative data using peptidoglycan (PG) which is structurally similar to the peptidoglycan used in claim 1 of Yabiki. These data show that peptidoglycan is less effective than the inventive LPS in stimulating the immune defense mechanism. Applicants further submit that with respect to Rorstad, it would be difficult for one skilled in the art to obtain any comparative data using the glucan of Rorstad since appreciable amounts of the compound are not obtainable.

In view of the foregoing, Applicants submit that the Examiner's rejection has been obviated and overcome.

VIII. Response to Rejection of Claims 1-12, 15 and 16 under 35 U.S.C. § 103(a)

Claims 1-12, 15 and 16 are rejected under 35 U.S.C. § 103(a) over Takahashi, in view of Matsuyama and further in view of Soma et al.

The Examiner considers the instant claims *prima facia* obvious over Takahashi, Matsuyama and Soma, since Takahashi teaches a fungal-derived glycan to protect against infectious disease in crustaceans, Matsuyama teaches treatment of streptococcal infection in fish, specifically Yellowtails with fungal-derived glucan, and Soma teaches Pantoea agglomerans and its lipopolysaccharide with a low molecular weight for stimulating immunity.

The Examiner alleges that it would have been obvious to combine the low weight lipopolysaccharide of Soma with those references teaching the addition of prophylactic

substances in feed for fish and crustaceans.

Applicants traverse for the following reasons.

Applicants submit that the Examiner has reached this conclusion through hindsight analysis and is merely combining elements taught in the prior art without any teaching in those references which would suggest the desirability of their combination. As such, the Examiner has failed to establish a *prima facie* case of obviousness and these references are an improper basis for rejection under § 103.

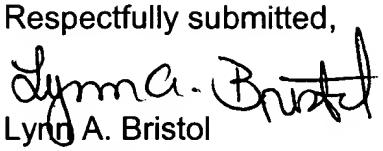
The Examiner's attention is further directed to Example 5 of the present specification, more specifically Group 6 which demonstrates comparative data using the glycan derived from the fungus, Schizophyllum commune (JP-B-6-65649) which is the same molecule as the glucan of Takahashi. These data show that the glucan is less effective than the inventive LPS in stimulating the immune defense mechanism. Applicants further submit that with respect to Matsuyama, it would be difficult for one skilled in the art to obtain any comparative data using the glucan of Matsuyama since appreciable amounts of the compound are not obtainable.

CONCLUSIONS

Applicants submit that in view of the foregoing amended title, specification and claims, the Examiner's objections have been met and overcome. Additionally, in view of the amended claims and all of the foregoing arguments, the Examiner's rejections of the claims under 35 U.S.C. § 101, 102(b), 103(a) and 112, first and second paragraph, have been met and overcome. Applicants respectfully submit that the claims are now in condition for allowance, and that the Examiner allow the application to pass to issuance.

In the event any fees are required, please charge our Deposit Account No. 01-2300.

Respectfully submitted,



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3. (Amended) [Use of the low molecular weight lipopolysaccharide of claim 1 for the preparation of a feedstuff additive for crustaceans or fishes] A method for preparing a feedstuff additive for crustaceans or fishes comprising

a) incubating a gram negative bacterium in culture medium;

b) collecting the bacterium from cultured medium of step a);

c) extracting the bacterium to obtain an aqueous extract;

d) applying the aqueous extract over an anion exchange resin for obtaining a purified feedstuff additive.

5. (Amended) An agent for [preventing the perish] activating immunity or preventing infection of crustaceans or fishes comprising the low molecular weight lipopolysaccharide of claim 1 as [in] an effective component and a carrier.

6. (Amended) An agent for [preventing the perish] activating immunity or preventing infection of crustaceans or fishes comprising an admixture of an immunity-activating or infection-preventing amount of the low molecular weight lipopolysaccharide of claim 1 and a [carrier acceptable for crustaceans and fishes] feed.

15. (Amended) A feedstuff additive according to claim 1, wherein the [infectious diseases] infection is caused by an infectious microbe selected from the group consisting of: [is] acute viremia of crustaceans, their vivrio diseases, parasitosis or mycosis; iridovixus infectious diseases of fishes, their rhabdovirus diseases, neuronecrosis, infectious hemopoietic organ necrosis, pseudotuberculosis, [streptococcic] streptococcal diseases, enterococcus diseases, vivrio diseases, cold-water disease, Pseudomonas diseases, gliding-bacteria diseases [or] and

Saprolegnia diseases.

Substitute Spec (NE)

#11

JM

12/28/01

SPECIFICATION

1

ADDITIVES FOR CRUSTACEAN OR FISH FEEDS AND FEEDS

OK to Enter KS

TECHNICAL FIELD

[0001] The present invention relates to a feedstuff additive for crustaceans or fishes, and a feed containing the feedstuff additive, and more particularly to a feedstuff additive which shows significant effects of activating immunity and preventing infection and to a feed containing the same in a suitable proportion.

BACKGROUND ART

[0002] In recent years, there has been a significant development in aquiculture techniques for crustaceans and fishes. Great economic damage in the culture industry is due to outbreaks of bacterial or viral diseases of crustaceans and fishes. Diseases of crustaceans and fishes often occurring include acute viremia of kuruma prawns (Penaeus japonicus), vibriosis thereof, pseudotuberculosis of yellowtails, enterococcus diseases thereof, cold-water disease of sweet fishes (ayu), Pseudomonas diseases thereof, iridovirus diseases of red sea breams, Seriola dumerili, yellowtails or the like. Of these diseases, bacterial diseases have been treated with antibiotics or synthetic antibacterial agents as a curative agent. However, with the advent of antibiotic-resistant bacteria, satisfactory curative effects have not been achieved. Public health hazards are also an issue because of residual

amounts of medicinal agents remaining in crustaceans and fishes. Consequently, there is a strong demand for preventive measures that do not rely on chemotherapy. On the other hand, vaccines and curative agents have not been developed against viral diseases of crustaceans and fishes, and viral diseases still often occur.

[0003] The use of polysaccharides is already known to have an immunopotentiating effect on crustaceans and fishes, and to prevent infectious diseases thereof. These polysaccharides include, for example, peptidoglycan derived from Bifidobacterium thermophilum (Patent No.2547371), cell wall-forming component of gram-positive bacteria like bacteria of genus Bacillus (JP-B-3-173826) and β-1,3-glucan derived from Schizophyllum commune (JP-B-6-65649). It was already reported that high molecular weight lipopolysaccharides activate the immune function of fishes and animals (Salati, F. and R. Kusuda, Society Journal, Japanese Society of Science of Fisheries, vol.53, pp.201 to 204, 1987 and Odean, M.J. et al., Infection and Immunity, vol.58, pp.427 to 432, 1990).

[0004] On the other hand, the low molecular weight lipopolysaccharide of the present invention (hereinafter referred to as "low molecular weight LPS") is different in basic structure and components from the peptidoglycan derived from gram-positive bacteria, cell wall-forming component and β-1,3-glucan derived from a mushroom. The low molecular weight LPS of the invention comprises three components, i.e. a specific lipid A, an oligosaccharide with covalent bond therewith called R core and O specific polysaccharide. The low

molecular weight LPS of the invention is well known as an immunopotentiator for animals because of its ability to induce tumor necrosis factor (TNF)expression, but until the present invention, it was not known that LPS could prevent infectious diseases in crustaceans and fishes. The high molecular weight lipopolysaccharides (LPSs) used in previous studies are characterized in having a markedly high molecular weight, i.e., 1 million to 10 million, and high toxicity. Consequently, when administered to crustaceans and fishes over long periods of time, such high molecular weight LPS is unable to sustain an activated immune function. High molecular weight forms of LPS must be orally administered in a large quantity because of their poor intestinal absorption. Consequently, a prolonged administration of LPS frequently results in impaired immune function.

[0005] A variety of infectious diseases often occur in crustaceans and fishes. Some of these diseases are lethal and may result in great economic and commercial loss. A disadvantage of aquaculture techniques is that the immune function of crustaceans and fishes is compromised as a result of breeding in overcrowded areas under limited environmental conditions. Various substances have been used to reactivate the impaired immune systems of aquaculture-derived crustaceans and fishes. On the other hand, crustaceans are incapable of producing antibodies, lymphocytes, neutrophils and basophils as found in vertebrates. Fishes have a limited ability to produce an antibody and its production of antibody is greatly affected by the temperature of water because they are cold-blooded

animals. In other words, substantial differences exist in the defense mechanisms between oceanic organisms and mammals (Fish Pathology, 30(2), 141-150, June in 1995). Consequently some substances are not usable, in-situ, in breeding oceanic organisms because of problems associated with high toxicity such as LPSs.

[0006] An object of the present invention is to provide a safe feedstuff additive for culturing or breeding of crustaceans and fishes. The feedstuff additive can prevent infectious diseases even in small amounts by activating the intrinsic immune function. The inventive LPS is free from problems of public health hazards such as other feedstuff additives which are not metabolized and which accumulate in crustaceans and fishes.

DETAILED DESCRIPTION OF THE INVENTION

[0007] The present invention relates to a feedstuff additive for crustaceans and fishes, characterized in that it is prepared from gram-negative bacteria, that it has a molecular weight of 5000 ± 2000 as measured by SDS-PAGE method using a protein marker, that it is substantially free of high molecular weight lipopolysaccharide, and that it contains a low molecular weight lipopolysaccharide as an effective component and that it is capable of activating immunity or preventing infection in crustaceans or fishes.

[0008] The invention also relates to a feed for crustaceans or fishes wherein the feed is characterized in that it contains the feedstuff additive.

[0009] The present invention also provides a feedstuff additive for crustaceans or fishes comprising the low molecular weight lipopolysaccharide and a carrier acceptable for crustaceans and fishes.

[0010] The present invention also provides use of the low molecular weight lipopolysaccharide for the preparation of a feedstuff additive for crustaceans or fishes.

[0011] The present invention also provides a method of activating immunity or preventing infection in crustaceans and fishes comprising administering an effective amount of the low molecular weight lipopolysaccharide to crustaceans or fishes.

[0012] The present invention also provides an agent for prolonging survival of crustaceans or fishes comprising the low molecular weight lipopolysaccharide as an effective component.

[0013] The present invention also provides an agent for prolonging survival of crustaceans or fishes comprising the low molecular weight lipopolysaccharide and a carrier acceptable for crustaceans and fishes.

[0014] The present invention also provides use of the low molecular weight lipopolysaccharide of for the preparation of an agent for prolonging survival of crustaceans or fishes.

[0015] The present invention also provides a method of prolonging survival of crustaceans or fishes comprising administering an effective amount of the low molecular weight lipopolysacchride to crustaceans or fishes.

[0016] The present invention also provides a feedstuff additive, wherein the gram-negative bacteria are those pertaining to genus Pantoea.

[0017] The present invention also provides a feedstuff additive, wherein the gram-negative bacteria are Pantoea agglomerans.

[0018] The present invention also provides a feed for crustaceans or fishes comprising the feedstuff additive.

[0019] The present invention also provides a feed for crustaceans or fishes comprising the agent for prolonging survival.

[0020] The present invention also provides a method of breeding crustaceans or fishes comprising administering the feed to crustaceans or fishes.

[0021] The feedstuff additive of the invention is prepared from gram-negative bacteria by purification according to the method disclosed in JP-A-8-198902. The present inventors prepared a feed containing a low molecular weight LPS having a molecular weight of 5000 ± 2000. When the feed was supplied to crustaceans and fishes, it was found that the feed prevented viral or bacterial infectious diseases and protected them against decease by activation of the intrinsic immune function.

[0022] The low molecular weight LPS of the present invention is, as described above, a lipopolysaccharide having a molecular weight of 5000 ± 2000 which is prepared from gram-negative bacteria according to the method disclosed in JP-A-8-198902. The LPS of the invention is significantly safer for crustaceans or fishes, and produces significantly improved effects on activating immunity, in preventing infection, and prolonging survival compared to conventional LPSs (with a molecular weight

of 1 million to 10 millions).

[0023] In the present invention, the term "substantially free of high molecular weight lipopolysaccharide" means "not containing lipopolysaccharide having a molecular weight of at least 8,000".

[0024] The gram-negative bacteria from which the inventive LPS can be derived includes but is not limited to the genera for Pantoea, Salmonella, Aeromonas, Serratia and Enterobacter, and further include those described in JP-A-4-99481. Pantoea are preferred, and those of Pantoea agglomerans are most preferred.

[0025] The low molecular weight LPS of the present invention can be prepared by a method comprising incubating gram-negative bacteria in a conventional manner, collecting the cultured bacteria from the culture medium, extracting the collected bacteria by conventional methods, such as hot phenol method (edited by O. Westphal, Methods in Carbohydrate Chemistry, vol. 5, p.83, Academic Press, 1965) and purifying the extract with an anion exchange resin. More specifically, the method comprises suspending bacteria in distilled water, adding the suspension to a mixture of distilled water and an equal volume of hot phenol, stirring the mixture, centrifuging the mixture to recover the aqueous layer, dialyzing the aqueous layer to remove the phenol, concentrating the aqueous layer by ultrafiltration to obtain crude LPS fractions, purifying the fractions by conventional anion exchange chromatography (e.g. using mono Q-Sepharose or Q-Sepharose) and desalting the same in the conventional manner.

[0026] The purified LPS thus obtained is substantially identical with the LPSs having a molecular weight of about 5,000 to about 6,000 as disclosed in JP-A-4-187640, JP-A-4-49240, JP-A-4-99481 and JP-A-5-155778. The purified LPS is subjected to gel filtration in the presence of a surface-active agent such as sodium deoxycholate to recover only low molecular weight LPS-containing fractions, whereby only a highly purified low molecular weight LPS is obtained by removal of the high molecular weight LPS from the fractions. The procedure of gel filtration in the presence of a surface-active agent is carried out to more highly purify the LPSs having a molecular weight of about 5,000 to about 6,000 which are disclosed in JP-A-4-187640, JP-A-4-49240 and JP-A-5-155778, whereby the high molecular weight LPS is completely removed from the fractions.

[0027] The term "crustaceans" used herein refers to all of lobsters, shrimps or prawns such as kuruma prawn (Penaeus japonicus), ushi prawn (Penaeus monodon), Yellow Sea prawn (Penaeus chinensis) and banana prawn (Penaeus morguiensis), and all crabs such as Portunus trituberculatus and Chinese mitten crab, preferably lobsters, shrimps or prawns, more preferably prawns.

[0028] The term "fishes" used herein include all fishes such as yellowtail, globefish, real sea bream, flatfish, eel and rainbow trout.

[0029] The infectious diseases referred to herein include acute viremia of crustaceans, their vivrio diseases, parasitosis such as Bpistylis sp. or Zoothamnium sp., or mycosis such as Lagenidium sp. or Siropidium sp.; iridovirus

infectious diseases of fishes, their rhabdovirus diseases, neuronecrosis, infectious hemopoietic organ necrosis, pseudotuberculosis, streptococcal diseases, enterococcal diseases, vivrio diseases, cold-water disease, Pseudomonal diseases, gliding-bacteria diseases and Saprolegnia diseases, and all of infectious diseases caused by viruses, mycoplasmas, bacteria, fungi and parasites, among which the feedstuff additive and feed of the invention can be more effectively used for viremia of crustaceans, and fishes' diseases such as streptococcal diseases, enterococcal diseases and vivrio diseases.

[0030] The low molecular weight LPS of the present invention can be used as a feed additive for crustaceans and fishes, alone or in combination with conventional carriers, stabilizers and the like, and optionally with vitamins, amino acids, minerals and like nutrients, antioxidants, antibiotics, antibacterial agents and other additives. The feed additive is prepared in a suitable form such as powders, granules, pellets or suspensions. The feed additive may be supplied to crustaceans or fishes, alone or in combination with a feed. For prevention of diseases, the feed additive may be supplied together with the feed ad libitum or at determined time periods.

[0031] The feeds of the present invention are not specifically limited but can be any of powdery feeds, solid feeds, moist pellet feeds, dry pellet feeds, extruder pellet feeds and live baits.

[0032] The concentration of the low molecular weight LPS in the feed of the invention can be selected from a wide range,

and is preferably 0.000001 to 0.001% by weight, more preferably 0.00002 to 0.00005% by weight to which its proportion is not limited. The dose amount of the low molecular weight LPS can be determined for each of the crustaceans or fishes as needed. For example, the LPS is administered at a daily dose of 1 to 100 μg, preferably 10 to 20 μg, per kilogram of the body weight of crustaceans or fishes to which, however, the dose is not limited.

[0033] The present invention will be described in detail with reference to the following Examples to which, however, the invention is not limited.

EXAMPLES

[0034] Low molecular weight LPS used in the Examples is LPS having a molecular weight of about 5,000, and high molecular weight LPS is LPS having a molecular weight of about 8,000 to 50,000.

Reference Example 1 (Preparation of low molecular weight LPS)

[0035] A 10 g quantity of tryptone (product of DIFCO CO.), 5 g of yeast extract (product of DIFCO CO.) and 10 g of NaCl (product of WAKO PURE CHEMICAL INDUSTRIES, LTD., special grade) were added to 1 liter of distilled water. The suspension was adjusted to a pH of 7.5 with NaOH and was sterilized in an autoclave. A single colony was separated from Pantoea agglomerance-carrying bacteria maintained at -80°C and was inoculated in a 500 ml-vol. Sakaguchi flask holding 100 ml of a culture medium containing sterile glucose (product of WAKO PURE CHEMICAL INDUSTRIES, LTD., special grade) at a proportion

of 0.1% (hereinafter referred to as L-broth medium). The cells were subjected to shake culture at 35°C overnight. The cultured cells were inoculated into a 3 liter-vol. Sakaguchi flask holding 1,000 ml of L-broth medium, and were further cultivated in the same manner as above.

[0036] The cultured cells were inoculated in a 10-liter vol. desk fermenter (product of MARUBISHI BIOENGI CO.) holding 7 liters of L-broth medium, and were subjected to aeration culture under the same conditions. The cells were collected to recover about 70 g of wet bacteria and were freeze-stored. About 70 g of freeze-stored cells were suspended in 500 ml of distilled water. A 500-ml quantity of 90% hot phenol was added to the suspension. The mixture was stirred at 65 to 70°C for 20 minutes and was cooled. The mixture was centrifuged at 10,000 G and 4°C for 20 minutes to recover the aqueous layer. The phenol layer was treated in the same manner as above. Then the two aqueous layers thus obtained were combined and dialyzed overnight to remove the phenol. The inner solution was concentrated by ultrafiltration under nitrogen gas using an ultrafiltration device (product of ADVANTEC TOYO CO., K-200) with a membrane filter 200,000 for molecular weight cut-off.

[0037] The lyophilized product of crude LPS thus obtained was dissolved in distilled water, the filter was sterilized, a buffer was added, and the solution was subjected to anion exchange chromatography (product of PHARMACIA Co., Q-Sepharose first flow). The specimen solution was passed through the column using a buffer containing 10 mM Tris-HCl (pH 7.5) and 10 mM NaCl to elute a limulus active fraction with 200 to 400 mM

NaCl/10 mM Tris-HCL (pH 7.5). The eluate was subjected to ultrafiltration under the same conditions as above for desalting and concentration, and was lyophilized to obtain about 300 mg of purified LPS from about 70 g of wet bacteria.

[0038] The purified LPS (100 mg) was dissolved in a solubilizing buffer [comprising 3% sodium deoxycholate (product of WAKO PURE CHEMICAL INDUSTRIES LTD.), 0.2 M sodium chloride, 5 mM EDTA-2Na and 20 mM Tris-hydrochloric acid, pH 8.3]. The purified LPS solution (20 ml) was gently placed over a Sephacryl S-200 HR column (product of PHARMACIA CO.). Then, 800 ml of the solution was eluted with an eluting buffer [comprising 0.25% sodium deoxycholate (product of WAKO PURE CHEMICAL INDUSTRIES LTD.), 0.2 M sodium chloride, 5 mM EDTA and 10 mM Tris hydrochoric acid, pH 8.3] at a flow velocity of 16 ml/hr for 50 hours.

[0039] The eluate was fractionated by a fraction collector (product of ADVANTEC CO., trade name SF 2120) under control of flow velocity using a perista-pump PI (product of PHARMACIA CO.). The initial 240-ml portion (24- fraction portion) was discarded. Thereafter, the residue was fractionated into 80 fractions at 10 ml/fraction. The saccharide in the eluted fractions was quantitated using the base solution or diluted solution by phenol/sulfuric acid method (Sakuzo FUKUI, "Method of Quantitative Determination of Reducing Sugar", 2nd ed., pp. 50 to 52, Gakkai Shuppan Center, 1990) to check the elution state. The fraction pattern of LPS was investigated by SDS-PAGE method using 0.5 ml of each of fractions 37 to 55 among the fractions presumably having LPS (fractions 30 to 60).

[0040] The result of this investigation demonstrates that fractions 45 to 55 contained only low molecular weight LPS (m.w. about 5000), and that fractions 37 to 44 contained both low molecular weight LPS and high molecular weight LPS. The low molecular weight LPS fractions of fractions 45 to 55 were further purified as follows.

[0041] The fractions were mixed, lyophilized and suspended in ethanol. The suspension was centrifuged to remove the deoxycholic acid soluble in ethanol, and to recover a low molecular weight LPS in insoluble fractions. The ethanol treatment of the low molecular weight LPS fractions was further repeated twice, followed by removal of deoxycholic acid. The LPS was suspended in 70% ethanol, and the buffer component was removed by centrifugation. The same procedure was repeated three times for recovery of low molecular weight LPS in the insoluble fractions, followed by lyophilization, whereby about 20 mg of purified low molecular weight LPS was produced.

Example 1 (Safety of low molecular weight LPS in crustaceans)

[0042] Kuruma prawns having an average weight of 20 g were divided into 5 groups of 20 prawns each. The low molecular weight LPS of the present invention was intramascularly administered to the third abdominal segment of prawns in Groups 1 and 2 at a dose of 50 mg and 100 mg, respectively per kilogram of the prawn's weight. On the other hand, a conventional high molecular weight LPS (derived from E. coli, E. coli 0111 manufactured by DIFCO CO.) was intramascularly administered to the third abdominal segment of prawns in Groups 3 and 4 at a dose of 10 mg and 20 mg, respectively per kilogram

of the prawn's weight. Group 5 received a physiological saline free of LPS. The life or death of prawns up to 120 hours after administration was checked to determine mortality. The results are shown in Table 1.

Table 1

Group	deaths number tested	mortality rate (%)
Group 1 low MW LPS 50 mg/kg	0/20	0
Group 2 low MW LPS 100 mg/kg	0/20	0
Group 3 high MW LPS 10 mg/kg	13/20	65
Group 4 high MW LPS 20 mg/kg	20/20	100
Group 5 physiological saline	0/20	0

[0043] As shown in Table 1, the mortality rate of prawns in the groups receiving 10 mg or 20 mg of high molecular weight LPS was 65 or 100%, respectively, whereas no prawns died in the groups receiving 50 mg and 100 mg of low molecular weight LPS. It is clear from the above data that low molecular weight LPSs are significantly safe for prawns as compared with conventional high molecular weight LPSs.

Example 2 (Safety of low molecular weight LPS in fishes)

[0044] Black carp having an average weight of 85 g, were divided into 3 groups of 40 carp each. The low molecular weight LPS of the present invention was intramascularly administered to the dorsal region of black carp in Group 1 at a dose of 100 mg per kilogram of the carp's weight. On the other hand, a conventional high molecular weight LPS (trade name E. coli 0111 manufactured by DIFCO CO.) was

intramascularly administered to the dorsal region of black carp in Group 2 at a dose of 20 mg per kilogram of the carp's weight. Group 3 received a physiological saline free of LPS. The viability of black carp was evaluated up to 120 hours after administration. The results are shown in Table 2.

Table 2

Group	deaths number tested	mortality rate (%)
Group 1 low MW LPS 100 mg/kg	0/40	0
Group 2 high MW LPS 20 mg/kg	34/40	85
Group 3 physiological saline	0/40	0

[0045] As shown in Table 2, the mortality of black carp was 85% in the group receiving 20 mg of high molecular weight LPS, whereas no black carp died in the group receiving 100 mg of low molecular weight LPS. It is clear from the above data that the low molecular weight LPS of the present invention is significantly safe for fishes as compared with conventional high molecular weight LPS.

Example 3 (Activation of phagocytosis in hemocytes of crustaceans)

[0046] Kuruma prawns having an average weight of 20 g were divided into 6 groups of 20 prawns each. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention admixed with feeds at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of each prawn's weight. On the other hand, Group 4 received a high molecular weight LPS admixed with a feed at a daily dose of 100 μg, and Group 5

received the same at a daily dose of 1000 μg, per kilogram of each prawn's weight. The feeds were given for 7 days. Group 6 was given a feed free of LPS. On days 0, 1, 5 and 7, the blood was collected from the thorax recess of prawns using a syringe holding a K-199 culture medium containing L-cystein as an anticoagulant. Hemocyte cells were obtained by centrifugation. The cells (1×10^5 cells per microliter of the suspension) were mixed with 1×10^8 latex beads (1.986 μm in diameter), and reacted at 25°C for 30 minutes. After fixing the reaction mixture with glutaraldehyde, it was air-dried. The mixture was giemsa stained and fixed to a glass slide with EUKITT (mounting medium; O. Kindler GmbH & Co., Freiburg, Germany). The same procedure was repeated to obtain five samples per prawn. The hemocyte cells (200 cells per sample) were observed at random under an epi-fluorescent microscope to determine the phagocytic index after LPS stimulation. The phagocytic index was calculated by the following equation:

Phagocytosis ratio=[number of hemocyte cells taking beads/total number of hemocyte cells observed] $\times$ 100.

Average number of beads phagocytosed by hemocyte cells = number of beads phagocytosed by hemocyte cells/number of hemocyte cells with phagocytosed beads.

Phagocytosis index=phagocytosis ratio X average number of beads phagocytosed by hemocyte cells X 100.

Test results: The biophylaxis of crustaceans involves a cell factor component and a liquid factor component. The phagocytosis of foreign particles in hemocytes is associated with the cellular compartment. Phagocytosis of foreign

particles by prawn hemocytes is an index that the immune defensive mechanism of a prawn is activated. [Yukinori TAKAHASHI et al, Research of Fish Diseases, 30 (2), pp.141 to 150, (1995)]. On this basis, the phagocytosis index was determined on days 0, 1, 5 and 7 after administration of feeds for the groups receiving high molecular weight LPSs and the groups receiving the low molecular weight LPSs. The results are shown in Table 3.

Table 3

Group	Phagocytosis index of hemocyte 0	1 day
Group 1 low MW LPS 20 μg/kg	0.9±0.18	2.1±0.61 *2
Group 2 low MW LPS 40μg/kg	0.9±0.18	3.3±1.16 *2
Group 3 low MW LPS 100μg/kg	0.9±0.18	3.8±1.00 *2
Group 4 high MW LPS 100μg/kg	0.9±0.18	0.7±0.31
Group 5 high MW LPS 1000μg/kg	0.9±0.18	1.1±0.63
Group 6 feed free of LPS	0.9±0.18	0.5±0.24

Group	Phagocytosis index of hemocyte 5 days	7 days
Group 1 low MW LPS 20 μg/kg	3.2±0.71 *2	8.4±1.37 *2
Group 2 low MW LPS 40μg/kg	4.5±0.75 *2	3.7±1.02 *2
Group 3 low MW LPS 100μg/kg	3.1±0.94 *2	2.8±0.70 *1
Group 4 high MW LPS 100μg/kg	0.7±0.82	1.2±0.44
Group 5 high MW LPS 1000μg/kg	2.1±0.58 *1	2.9±0.68 *1
Group 6 feed free of LPS	0.7±0.5	1.1±0.56

*1: Significant difference between this group and Group 6 ($P<0.05$)

*2: Significant difference between this group and Group 6

($P<0.01$)

[0047] Table 3 shows that the groups receiving the low molecular weight LPSs exhibited not only a higher phagocytosis index in hemocytes of prawns than Group 6, but a significant difference for this index compared to Group 6 ($P<0.01$, $P<0.05$). The group receiving 100 μg of conventional high molecular weight LPS was unable to increase the phagocytosis index in hemocytes of prawns after 1, 5 and 7 days. However, the group receiving 1000 μg of conventional high molecular weight LPS showed a significantly higher phagocytosis index in hemocytes of prawns $P<0.05$) compared to Group 6 after 5 and 7 days. These data show that the low molecular weight LPSs of the present invention can activate an immune defensive mechanism such as phagocytosis in hemocytes of prawns, even when used at a much lower concentration than the high molecular weight LPSs.

Example 4 (Induction of phenol oxidase in hemocytes of crustaceans)

[0048] Kuruma prawns having an average weight of 20 g were divided into 6 groups of 20 prawns each. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention admixed with feeds at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of prawn's weight. Group 4 received a high molecular weight LPS admixed with a feed at a daily dose of 100 μg, and Group 5 received the same admixed with a feed at a daily dose of 1000 μg, per kilogram of each prawn's weight. The administration of the feeds continued for 7 days. Group 6 received a LPS-free feed. The blood was collected from the thorax recess of prawns using a syringe containing KHE

culture medium with EDTA on days 0, 1, 5 and 7 after administration of feeds. The collected blood was centrifuged to obtain hemocyte cells. The cells were suspended in a Ca-Mg Hepes culture medium at a concentration of 1 X 10^6 cells/ml. The cells were lysed by freeze resolution and supersonic waves. The supernatant was separated by centrifugation and filtered with a membrane filter. The filtrate (900 μl) was mixed with 100 μl of L-DOPA solution as a substrate solution. Thereafter the mixture was reacted at a temperature of 60°C for 60 minutes. The absorbance at 490 nm was measured by a spectrophotometer to assess a phenol oxidase activity (PO activity).

Test results: The biophylaxis of crustaceans involves a cell factor component and a liquid factor component. The PO activity in hemocytes is associated with the cellular component. PO activity by prawn hemocytes is an index that the immune system is activated. The PO activity of prawns was determined on days 0, 1, 5 and 7 after administration of feeds for the groups receiving the low molecular weight LPSs and the groups receiving high molecular weight LPSs. The results are shown in Table 4.

Table 4

Group	PO activity (absorbance·490nm) 0	1 day	5 days	7 days
Group 1 low MW LPS 20 μg/kg	0.092	0.105	0.199 *1	0.405 *2
Group 2 low MW LPS 40μg/kg	0.092	0.115	0.201 *1	0.325 *2
Group 3 low MW LPS 100μg/kg	0.092	0.166 *1	0.170 *1	0.292 *2
Group 4 high MW LPS 100μg/kg	0.092	0.093	0.124	0.138

Group 5 high MW LPS 1000 μg/kg	0.092	0.104	0.197 *1	0.230 *1
Group 6 feed free of LPS	0.092	0.093	0.136	0.123

*1: significant difference between this group and Group 6 ($P<0.05$)

*2: significant difference between this group and Group 6 ($P<0.01$)

[0049] As shown in Table 4, the groups receiving the low molecular weight LPSs (present invention) exhibited not only a higher PO activity than Group 6 but a significant difference in this activity from Group 6 ($P<0.01$, $P<0.05$). The group receiving 100 μg of conventional high molecular weight LPS did not exhibit increased PO activity in hemocytes of prawns up to 7 days. The group receiving 1000 μg of conventional high molecular weight LPS showed a significantly higher PO activity in hemocytes of prawns($P<0.05$) than Group 6 after 5 and 7 days. These data show that the low molecular weight LPSs of the present invention can activate the immune defensive mechanism such as PO activity in hemocytes of prawns even when used at a much lower concentration than the high molecular weight LPSs.

Example 5 (Prevention of acute viremia in kuruma prawns)

[0050] Kuruma prawns having an average weight of 14 g were divided into 5 groups of 20 prawns each. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention admixed with feeds at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of each prawn's weight. Group 4

received a high molecular weight LPS admixed with a feed at a daily dose of 1000 μg, per kilogram of each prawn's weight. Group 5 received peptidoglycan (PG) derived from Bifidobacterium thermophilum (Patent No.2547371) admixed with a feed at a daily dose of 0.2mg (200 μg) per kilogram of each prawn's weight. Group 6 received β-1,3-glucan (1,3-G) derived from Schizophyllum commune (JP-B-6-65649) admixed with a feed at a daily dose of 50mg (50000 μg) per kilogram of each prawn's weight. The administration of feeds continued for 18 days. Group 7 (control group) was given an LPS-free feed.

[0051] On day 8 after the start of administration of LPS, an infection test was conducted using penaeid rod-shaped DNA virus (PRDV)as a pathogen for inducing acute viremia in prawns. Carapaces were removed from the cephalothorax of three prawns which died of acute viremia. The intestine of prawns was crushed and homogenized in 40 ml of sterile seawater. The supernatant (10 ml) was separated by centrifugation (10,000 × g, 10 minutes, 4°C) and added to 20 liters of seawater. On day 8 after the start of administration of LPS, prawns were infected with acute viremia by immersion in the supernatant for 2 hours. The viability of prawns was observed for 10 days after infection. The dead prawns were pathologically tested and examined by PCR (polymerase chain reaction) method to confirm whether the prawns died of PRDV infection.

Test results: Tables 5 and 6 show the total number of dead prawns and the mortality rate after infection with PRDV in the groups receiving low molecular weight LPSs of the present invention, the group receiving a high molecular weight LPS and

the group receiving a LPS-free feed.

Table 5

Group	Days after infection 1	2	3	4	5
Group 1 low MW LPS 20 μg/kg	0	0	0	2*	3
Group 2 low MW LPS 40 μg/kg	0	0	3	4	4
Group 3 low MW LPS 100 μg/kg	1	1	3	3	4
Group 4 high MW LPS 1000 μg/kg	1	1	6	6	6
Group 5 PG 0.2mg/kg	0	0	2	5	5
Group 6 1,3-G 50mg/kg	0	3	5	7	10
Group 7 feed free of LPS	2	4	13	14	15

* The number indicates the total number of dead prawns. (Other numbers show the same.)

Table 6

Group	Days after infection 6	7	8	9	10	Mortality
Group 1 low MW LPS 20 μg/kg	3	3	4	4	4	20 ***
Group 2 low MW LPS 40 μg/kg	6	6	6	7	7	35 ***
Group 3 low MW LPS 100 μg/kg	5	6	8	8	8	40 ***
Group 4 high MW LPS 1000 μg/kg	9	9	10	11	11	55 **
Group 5 PG 0.2mg/kg	7	8	8	8	10	50 **
Group 6 1,3-G 50mg/kg	10	11	11	12	12	60 **
Group 7 feed free of LPS	18	18	19	20	20	100

** : significant difference between this group and Group 7 ($P<0.05$)

*** : significant difference between this group and Group 7 ($P<0.01$)

[0052] All (100%) of prawns died in the control group receiving an LPS-free feed up to 9 days after infection with PRDV. On the other hand, 20%, 35% and 40% of prawns died in the groups receiving 20, 40 and 100 μg, respectively, of low molecular weight LPS (present invention). In other words, a low mortality rate was observed for these groups, and a significant difference ($P<0.01$) exists between these groups and the control group. In contrast, 55% of prawns died in the group receiving 1000 μg of high molecular weight LPS, which means that more prawns died in this group than the groups receiving the low molecular weight LPSs. The above data demonstrate that the low molecular weight LPSs of the present invention can prevent viral infection of prawns and that the low molecular weight LPSs are more efficacious than conventional high molecular weight LPSs.

Example 6 (Activation of immune function in fishes)

[0053] Yellowtails weighing 230 g on an average were divided into 6 groups of each 20 yellowtails. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention admixed with moist pellets at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of each yellowtail's weight. Group 4 received a high molecular weight LPS admixed with moist pellets at a daily dose of 100 μg, and Group 5 received a high molecular weight LPS admixed with moist pellets at a daily dose of 1000 μg, per kilogram of each yellowtail's weight. The

feeds were administered for 7 days. Group 6 received LPS-free moist pellets. On days 0, 1, 5 and 7 after administration of feeds, a head kidney was excised from 5 yellowtails. Hemocyte cells were separated in a plastic petri dish containing 0.25% NaCl RPMI-1640-HAH culture medium. The cells were passed through a cell filter to give a cell suspension. The suspension was placed over a discontinuous Percoll density gradient. Thereafter a leukocyte layer was formed by centrifugation (1600 rpm., at 4°C for 20 minutes).

[0054] The leukocyte layer was collected and was subjected to centrifugal washing after which the cells were suspended in a 10% FBS (fetal bovine serum)-containing 0.25% NaCl-including RPMI-1640-H culture medium. The number of leukocyte cells in the suspension was adjusted to 1 X 10^6 cells/ml. The leukocyte suspension (500 μl) and 500 μl of a suspension (1 $\times$ 10^8 cells/ml) of yeast opsonized with serum of yellowtail were placed into a silicone-treated glass test tube and were incubated at 25°C for 60 minutes with stirring every 10 minutes. After incubation, 5 smears per yellowtail were produced, subjected to Wright's staining and enclosed with EUKITT. The hemocyte cells (200 cells per smear) were observed at random under an optical microscope. The number of yeast cells phagocytized by leukocytes was counted. The phagocytosis index is the same as that in Example 3. The results are shown in Tables 7 and 8.

Table 7

Group	Phagocytosis index of leukocyte 0	1 day
Group 1 low MW LPS 20 μg/kg	7.3±2.30	12.7±2.65 *1
Group 2 low MW LPS 40μg/kg	7.3±2.30	17.9±3.99 *2
Group 3 low MW LPS 100μg/kg	7.3±2.30	18.6±4.12 *2
Group 4 high MW LPS 100μg/kg	7.3±2.30	6.3±2.24
Group 5 high MW LPS 1000μg/kg	7.3±2.30	8.2±2.18
Group 6 feed free of LPS	7.3±2.30	6.6±1.19

*1: significant difference between this group and Group 6 (P<0.05)

*2: significant difference between this group and Group 6 (P<0.01)

Table 8

Group	Phagocytosis index of leukocyte 5 days	7 days
Group 1 low MW LPS 20 μg/kg	39.2±2.54 *2	52.7±4.08 *2
Group 2 low MW LPS 40μg/kg	37.4±4.28 *2	37.0±3.11 *2
Group 3 low MW LPS 100μg/kg	42.6±5.35 *2	36.5±4.32 *1
Group 4 low MW LPS 100μg/kg	11.2±3.05	10.6±2.96
Group 5 low MW LPS 1000μg/kg	22.7±3.16 *1	31.8±3.52 *1
Group 6 feed free of LPS	9.0±2.04	7.7±1.73

*1: significant difference between this group and Group 6 (P<0.05)

**2: significant difference between this group and Group 6 (P<0.01)

[0055] As shown in Tables 7 and 8, any group of yellowtails receiving the low molecular weight LPSs (present invention) exhibited not only a higher phagocytosis index in leukocytes of yellowtails than Group 6, but also a significant difference ($P<0.01$, $P<0.05$) in this index compared to Group 6. However, the group receiving 100 μg of conventional high molecular weight LPS did not increase the phagocytosis index in leukocytes of yellowtails after 7 days. The group receiving 1000 μg of conventional high molecular weight LPS showed a significantly higher phagocytosis index ($P<0.01$) in leukocytes of yellowtails than Group 6 after 5 days. These data show that the low molecular weight LPSs of the present invention can activate the immune system of fishes such as phagocytosis in leukocytes at a lower concentration than conventional high molecular weight LPSs.

Example 7 (Prevention of Enterococcal disease in yellowtails)

[0056] Yellowtails weighing 63 g on an average were divided into 5 groups of 30 yellowtails each. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention admixed with moist pellets at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of each shrimp's weight. Group 4 received a high molecular weight LPS admixed with moist pellets at a daily dose of 1000 μg per kilogram of each yellowtail's weight. Group 5 (control) received LPS-free moist pellets. On day 7 after administration of feeds, the yellowtails were intraabdominally inoculated with Enterococcus Seriolicida at a concentration of 4.0×10^6 cells per yellowtail. The mortality rate 15 days after inoculation was determined. The results are

shown in Tables 9 and 10.

Table 9

Group	Days after infection 1	2	3	4	5	6	7	8	9
Group 1 low MW LPS 20 μg/kg	0	0	0	0	0	0	0	0	1*
Group 2 low MW LPS 40 μg/kg	0	0	0	1	1	2	2	4	4
Group 3 low MW LPS 100 μg/kg	0	0	0	0	0	1	3	3	5
Group 4 high MW LPS 1000 μg/kg	0	0	0	1	1	1	3	3	3
Group 5 feed free of LPS	0	0	1	2	7	7	10	12	16

* The number indicates the total number of dead yellowtails.

(Other numbers show the same.)

Table 10

Group	Days after infection 10	11	12	13	14	15	Mortality (%)
Group 1 low MW LPS 20 μg/kg	3	3	3	3	4	4	13.3 ***
Group 2 low MW LPS 40 μg/kg	7	8	8	8	8	8	26.7 **
Group 3 low MW LPS 100 μg/kg	5	5	5	7	7	7	23.3 **
Group 4 high MW LPS 1000 μg/kg	5	9	10	10	11	11	36.7 **
Group 5 feed free of LPS	16	16	17	22	22	22	73.3

** : significant difference between this group and Group 5 ($P<0.05$)

***: significant difference between this group and Group 5 ($P<0.01$)

[0057] Fifteen days after inoculation of E. Seriolicida, 73.3% of yellowtails died in the control group receiving LPS-

free feed. In contrast, a low mortality is indicated by the groups receiving the low molecular weight LPSs of the present invention, i.e. 13.3% from the group receiving 20 μg, 26.7% from the group receiving 40 μg and 23.3% from the group receiving 100 μg. In other words, there is a significant difference ($P<0.05$) in mortality between these groups and the control group. On the other hand, a mortality of 36.7% resulted from the group receiving 1000 μg of high molecular weight LPS. This group showed a higher mortality than the groups receiving low molecular weight LPSs. The above results show that the low molecular weight LPSs of the present invention can protect fishes against viral infection and are more efficacious than conventional high molecular weight LPSs.

INDUSTRIAL APPLICABILITY

[0058] The present invention provides a safe feedstuff additive for growing crustaceans and fishes, the feedstuff additive also capable of preventing infectious diseases by activating the intrinsic immune function of crustaceans and fishes, even when used in small amounts. The feedstuff additive is also effective in prolonging survival of crustaceans and fishes, and does not present a public health hazard because the LPS molecules do not accumulate at deleterious concentrations in crustaceans and fishes.

Marked-up copy of Specification

SPECIFICATION

ADDITIVES FOR CRUSTACEAN OR FISH FEEDS AND FEEDS

TECHNICAL FIELD

The present invention relates to a feedstuff additive for crustaceans or fishes, and a feed containing the feedstuff additive, and more particularly to a feedstuff additive which shows significant effects of activating immunity and preventing infection and to a feed containing the same in a suitable proportion.

BACKGROUND ART

In recent years, there has been a significant development in aquiculture techniques for crustaceans and fishes. Attendant on the development is a great economical damage in the culture industry due to outbreaks of bacterial or viral diseases of crustaceans and fishes. Diseases of crustaceans and fishes often occurring include acute viremia of kuruma prawns (Penaeus japonicus), vibriosis thereof, pseudotuberculosis of yellowtails, enterococcus diseases thereof, cold-water disease of sweet fishes (ayu), Pseudomonas diseases thereof, iridovirus diseases of red sea breams, Seriola dumerili, yellowtails or the like which have economically damaged the culture industry. Of these diseases, bacterial diseases have been treated with antibiotics or synthetic antibacterial agents as a curative agent. However, with the advent of antibiotic-resistant bacteria, satisfactory curative effects have not been achieved. Further, a problem of

Public health hazards has been raised because of the medicinal agents remaining in crustaceans and fishes. Consequently, there is a strong demand for preventive measures not depending on chemotherapy. On the other hand, vaccines and curative agents have not been developed against viral diseases of crustaceans and fishes, and viral diseases still often occur.

The use of polysaccharides is already known to immunopotentiate crustaceans and fishes, and to prevent infectious diseases thereof. These polysaccharides include, for example, peptidoglycan derived from Bifidobacterium thermophilum (Patent No.2547371), cell wall-forming component of gram-positive bacteria like bacteria of genus Bacillus (JP-B-3-173826) and β-1,3-glucan derived from Schizophyllum commune (JP-B-6-65649). It was already reported that high molecular weight lipopolysaccharides activate the immune function of fishes and animals (Salati, F. and R. Kusuda, Society Journal, Japanese Society of Science of Fisheries, vol.53, pp.201 to 204, 1987 and Odean, M.J. et al., Infection and Immunity, vol.58, pp.427 to 432, 1990).

On the other hand, the low molecular weight lipopolysaccharide of the present invention (hereinafter referred to as "low molecular weight LPS") is different in basic structure and components from the peptidoglycan derived from gram-positive bacteria, cell wall-forming component and β-1,3-glucan derived from a mushroom. The low molecular weight LPS of the invention comprises three components, i.e. a specific lipid A, an oligosaccharide with covalent bond therewith called R core and O specific polysaccharide. The low

molecular weight LPS of the invention is well known as an immunopotentiator for animals because of its ability to induce increase the tumor necrosis factor (TNF)-producing effect, but it was is not known at all to have an activity of preventing infectious diseases of in crustaceans and fishes. The high molecular weight lipopolysaccharides (LPSs) used in the researches heretofore reported are those with previous studies are characterized in having a markedly high molecular weight, as high as i.e., 1 million to 10 millions million and are of high toxicity. Consequently, when applied administered to crustaceans and fishes for a over long period, periods of time such high molecular weight LPS is unable to activate the immune function all the time. The sustain an activated immune function above-mentioned known substances have a high molecular weight forms of LPS and need to must be orally administered in a large quantity because of their poor intestinal absorption through the intestinal tract. Consequently, a long-period intake of them prolonged administration of LPS frequently results in impairment of immune function. impaired

As described above, A variety of infectious diseases often occur in crustaceans and fishes. Some of these diseases and commercial loss are lethal and may result in great economic damage. The A disadvantage of aquaculture techniques background to be noted is that the immune function of crustaceans and fishes is deteriorated because they are bred in compromised as a result breeding in an overcrowded area under a limited environment. environmental conditions Various substances were have used to reactivate their the impaired immune systems of aquaculture derived crustaceans and fishes. On the other hand, crustaceans have no ability to produce an are incapable of producing antibody nor lymphocytes, neutrophils or and basophils as found in a antibodies higher vertebrates. Fishes have a limited ability to produce an antibody and its production of antibody is greatly affected by the temperature of water because they are cold-blooded animals

so that such immune system is not sufficiently functioned. In other words, substantial differences exists in the defense mechanisms between these oceanic organisms and mammals (Fish Pathology, 30(2), 141-150, June in 1995). Consequently some of the substances are not usable in-situ in breeding oceanic organisms because of high toxicity like conventional LPSs such as, and most of them are impaired in the immune system by intake of the LPSs for a prolonged period.

problems associated with

An object of the present invention is to provide a safe feedstuff additive for culture or breeding culturing of crustaceans and fishes, the feedstuff additive being capable of preventing can prevent infectious diseases even in small amounts by properly activating their intrinsic immune function, and being free from problems of public health hazards such as the feedstuff additives remaining in crustaceans and fishes.

the

the inventive LPS is

other

which are not metabolized and which accumulate

DISCLOSURE OF THE INVENTION DETAILED DESCRIPTION OF THE INVENTION

The present invention provides relates to a feedstuff additive for crustaceans and fishes, characterized in that it is prepared from gram-negative bacteria, that it has a molecular weight of 5000 ± 2000 as measured by SDS-PAGE method using a protein marker, that it is substantially free of high molecular weight lipopolysaccharide, and that it contains a low molecular weight lipopolysaccharide as an effective component and that it is capable of activating immunity or preventing infection in crustaceans or fishes, and a feed for crustaceans or fishes which wherein the feed is characterized in that it contains the feedstuff additive.

A The invention also relates to

The present invention also provides a feedstuff additive for crustaceans or fishes comprising the low molecular weight lipopolysaccharide and a carrier acceptable for crustaceans and fishes.

The present invention also provides use of the low molecular weight lipopolysaccharide for the preparation of a feedstuff additive for crustaceans or fishes.

The present invention also provides a method of activating immunity or preventing infection in crustaceans and fishes comprising administering an effective amount of the low molecular weight lipopolysaccharide to crustaceans or fishes.

The present invention also provides an agent for [preventing the perish] prolonging survival of crustaceans or fishes comprising the low molecular weight lipopolysaccharide as an effective component.

The present invention also provides an agent for [preventing the perish] prolonging survival of crustaceans or fishes comprising the low molecular weight lipopolysaccharide and a carrier acceptable for crustaceans and fishes.

The present invention also provides use of the low molecular weight lipopolysaccharide of for the preparation of an agent for [preventing the perish] prolonging survival of crustaceans or fishes.

The present invention also provides a method of [preventing the perish] prolonging survival of crustaceans or fishes comprising administering an effective amount of the low molecular weight lipopolysacchride to crustaceans or fishes.

The present invention also provides a feedstuff additive, wherein the gram-negative bacteria are those pertaining to

genus Pantoea.

The present invention also provides a feedstuff additive, wherein the gram-negative bacteria are Pantoea agglomerans.

The present invention also provides a feed for crustaceans or fishes comprising the feedstuff additive.

The present invention also provides a feed for crustaceans or fishes comprising the agent for preventing the perish. prolonging survival

The present invention also provides a method of breeding crustaceans or fishes comprising administering the feed to crustaceans or fishes.

The feedstuff additive of the invention is prepared from gram-negative bacteria by purification e.g. according to the method disclosed in JP-A-8-198902. The present inventors prepared a feed containing a low molecular weight LPS having a molecular weight of 5000 ± 2000. When the feed was supplied to crustaceans and fishes, it was found that the feed prevented viral or bacterial infectious diseases and protected them against decease by activation of the intrinsic immune function. The present invention was accomplished based on this finding.

The low molecular weight LPS of the present invention is, as described above, a lipopolysaccharide having a molecular weight of 5000 ± 2000 which is prepared from gram-negative bacteria e.g. according to the method disclosed in JP-A-8-198902. The LPS of this invention is characterized in that the LPS is pronouncedly safer for crustaceans or fishes, and can produce a significantly higher effect of activating immunity, and a higher effect of preventing infection, and decease than prolonging survival

compared to
conventional LPSs (with a molecular weight of 1 million to 10 millions).

In the present invention, the term "substantially free of high molecular weight lipopolysaccharide" means "not containing lipopolysaccharide having a molecular weight of at least 8,000".

The gram-negative bacteria for use in the invention include, for example, those pertaining to genera Pantoea, Salmonella, Aeromonas, Serratia and Enterobacter, and further include those described in JP-A-4-99481. Among useful gram-negative bacteria, those of Pantoea are preferred, and those of Pantoea agglomerans are more preferred.

from which the inventive LPS can be derived includes but is not limited to the genera for

The low molecular weight LPS of the present invention can be prepared by a method comprising incubating gram-negative bacteria or the like in the conventional manner, collecting the cultured bacteria from the culture medium, extracting the collected bacteria by conventional methods, such as hot phenol method (edited by O. Westphal, Methods in Carbohydrate Chemistry, vol. 5, p.83, Academic Press, 1965) and purifying the extract with an anion exchange resin. More specifically, the method comprises suspending bacteria in distilled water, adding the suspension to a mixture of distilled water and an equal volume of hot phenol, stirring the mixture, centrifuging the mixture to recover the aqueous layer, dialyzing the aqueous layer to remove the phenol, concentrating the aqueous layer by ultrafiltration to obtain crude LPS fractions, purifying the fractions by conventional anion exchange chromatography (e.g. using mono Q-Sepharose or Q-Sepharose) and desalting the same in the conventional manner.

The purified LPS thus obtained is substantially identical with the LPSs having a molecular weight of about 5,000 to about 6,000 as disclosed in JP-A-4-187640, JP-A-4-49240, JP-A-4-99481 and JP-A-5-155778. The purified LPS is subjected to gel filtration in the presence of a surface-active agent such as sodium deoxycholate to recover only low molecular weight LPS-containing fractions, whereby only a highly purified low molecular weight LPS is obtained by removal of the high molecular weight LPS from the fractions. The procedure of gel filtration in the presence of a surface-active agent is carried out to more highly purify the LPSs having a molecular weight of about 5,000 to about 6,000 which are disclosed in JP-A-4-187640, JP-A-4-49240 and JP-A-5-155778, whereby the high molecular weight LPS is completely removed from the fractions.

The term "crustaceans" used herein refers to all of lobsters, shrimps or prawns such as kuruma prawn (Penaeus japonicus), ushi prawn (Penaeus monodon), Yellow Sea prawn (Penaeus chinensis) and banana prawn (Penaeus morguiensis), and all of crabs such as Portunus trituberculatus and Chinese mitten crab, preferably lobsters, shrimps or prawns, more preferably prawns. The term "fishes" used herein include all of fishes such as yellowtail, globefish, real sea bream, flatfish, eel and rainbow trout. The infectious diseases referred to herein include acute viremia of crustaceans, their vivrio diseases, parasitosis such as Bpistylis sp. or Zoothamnium sp. or mycosis such as Lagenidium sp. or Siropidium sp.; iridovirus infectious diseases of fishes, their rhabdovirus diseases, neuronecrosis, infectious hemopoietic organ necrosis,

pseudotuberculosis, streptococcic diseases, enterococcus diseases, vivrio diseases, cold-water disease, Pseudomonas diseases, gliding-bacteria diseases and Saprolegnia diseases, and all of infectious diseases caused by viruses, mycoplasmas, bacteria, fungi and parasites, among which the feedstuff additive and feed of the invention can be more effectively used for viremia of crustaceans, and fishes' diseases such as streptococcic diseases, enterococcus diseases and vivrio diseases.

The low molecular weight LPS of the present invention can be used as a feed additive for crustaceans and fishes, and for this purpose, may be used as it is or as mixed with conventional carriers, stabilizers and the like, and optionally with vitamins, amino acids, minerals and like nutrients, antioxidants, antibiotics, antibacterial agents and other additives. The feed additive is prepared in a suitable form such as powders, granules, pellets or suspensions. The feed additive may be supplied to crustaceans or fishes, alone or in mixture with a feed. For prevention of diseases, the feed additive may be supplied together with the feed at all times or at a latter half of feeding time.

The feeds of the present invention are not specifically limited but can be any of powdery feeds, solid feeds, moist pellet feeds, dry pellet feeds, extruder pellet feeds and live baits.

The proportion of the low molecular weight LPS in the feed of the invention can be selected from a wide range, and is preferably 0.000001 to 0.001% by weight, more preferably

0.00002 to 0.00005% by weight to which its proportion is not limited. The amount of the low molecular weight LPS to be used can be suitably determined. For example, the LPS is applied at a daily dose of 1 to 100 μg, preferably 10 to 20 μg, per kilogram of the body weight of crustaceans or fishes to which, however, the dose is not limited.

BEST MODE OF CARRYING OUT THE INVENTION

The present invention will be described in detail with reference to the following Examples to which, however, the invention is not limited. Low molecular weight LPS used in the Examples is LPS having a molecular weight of about 5,000, and high molecular weight LPS is LPS having a molecular weight of about 8,000 to 50,000.

Reference Example 1 (Preparation of low molecular weight LPS)

A 10 g quantity of tryptone (product of DIFCO CO.), 5 g of yeast extract (product of DIFCO CO.) and 10 g of NaCl (product of WAKO PURE CHEMICAL INDUSTRIES, LTD., special grade) were added to 1 liter of distilled water. The suspension was adjusted to a pH of 7.5 with NaOH and was sterilized in an autoclave. A single colony was separated from Pantoea agglomerance-carrying bacteria maintained at -80°C and was inoculated in a 500 ml-vol. Sakaguchi flask holding 100 ml of a culture medium containing sterile glucose (product of WAKO PURE CHEMICAL INDUSTRIES, LTD., special grade) at a proportion of 0.1% (hereinafter referred to as L-broth medium). Then the cells were subjected to shake culture at 35°C overnight. The cultured cells were inoculated in its entirety into a 3 liter-vol.

Sakaguchi flask holding 1,000 ml of L-broth medium, and were further cultivated in the same manner as above.

The cultured cells were inoculated in a 10-liter vol. desk fermenter (product of MARUBISHI BIOENGI CO.) holding 7 liters of L-broth medium, and were subjected to aeration culture under the same conditions. The cells were collected to recover about 70 g of wet bacteria and were freeze-stored. About 70 g of freeze-stored cells were suspended in 500 ml of distilled water. A 500-ml quantity of 90% hot phenol was added to the suspension. The mixture was stirred at 65 to 70°C for 20 minutes and was cooled. The mixture was centrifuged at 10,000 G and 4°C for 20 minutes to recover the aqueous layer. The phenol layer was treated in the same manner as above. Then the two aqueous layers thus obtained were combined and dialyzed overnight to remove the phenol. The inner solution was concentrated by ultrafiltration [in a 2 atom.] under nitrogen gas using an ultrafiltration device (product of ADVANTEC TOYO CO., K-200) with a membrane filter [by cutting off molecular weight] 200,000. for molecular weight cut-off.

The lyophilized product of crude LPS thus obtained was dissolved in distilled water, the filter was sterilized, a buffer was added, and the solution was subjected to anion exchange chromatography (product of PHARMACIA Co., Q-Sepharose first flow). The specimen solution was passed through the column using a buffer containing 10 mM Tris-HCl (pH 7.5) and 10 mM NaCl to elute a limulus active fraction with 200 to 400 mM NaCl/10 mM Tris-HCL (pH 7.5). The eluate was subjected to ultrafiltration under the same conditions as above for desalting and concentration, and was lyophilized to obtain about

300 mg of purified LPS from about 70 g of wet bacteria.

The obtained purified LPS (100 mg) was dissolved in a solubilizing buffer [comprising 3% sodium deoxycholate (product of WAKO PURE CHEMICAL INDUSTRIES LTD.), 0.2 M sodium chloride, 5 mM EDTA-2Na and 20 mM Tris-hydrochloric acid, pH 8.3]. The purified LPS solution (20 ml) was gently placed over a Sephacryl S-200 HR column (product of PHARMACIA CO.). Then, 800 ml (50 hours) of the solution was eluted with an eluting buffer [comprising 0.25% sodium deoxycholate (product of WAKO PURE CHEMICAL INDUSTRIES LTD.), 0.2 M sodium chloride, 5 mM EDTA and 10 mM Tris hydrochoric acid, pH 8.3] at a flow velocity of 16 ml/hr. for 50 hrs

The obtained eluate was fractionated by a fraction collector (product of ADVANTEC CO., trade name SF 2120) under control of flow velocity using a perista-pump PI (product of PHARMACIA CO.). A first 240-ml portion (24- fraction portion) was cast away! The initial discarded Thereafter, the residue was fractionated into 80 fractions at 10 ml/fraction. The saccharide in the eluted fractions was quantitatively determined quantitated using the base solution or diluted solution by phenol/sulfuric acid method (Sakuzo FUKUI, "Method of Quantitative Determination of Reducing Sugar", 2nd ed., pp. 50 to 52, Gakkai Shuppan Center, 1990) to check the elution state. The fraction pattern of LPS was investigated by SDS-PAGE method using 0.5 ml of each of fractions 37 to 55 among the fractions presumably having LPS (fractions 30 to 60).

The result of this investigation demonstrates that the fractions 45 to 55 contained only low molecular weight LPS

(m.w. about 5000), and that fractions 37 to 44 contained both low molecular weight LPS and high molecular weight LPS. The low molecular weight LPS fractions of fractions 45 to 55 were further purified as follows.

The fractions [was] were mixed, lyophilized and suspended in ethanol. The suspension was centrifuged to remove the deoxycholic acid soluble in ethanol, and to recover a low molecular weight LPS in insoluble fractions. The ethanol treatment of the low molecular weight LPS fractions was further repeated twice, followed by removal of deoxycholic acid. The [obtained] LPS was suspended in 70% ethanol [again], and the buffer component was removed by centrifugation. The same procedure was repeated three times for recovery of low molecular weight LPS in the insoluble fractions, followed by lyophilization, whereby about 20 mg of purified low molecular weight LPS was produced.

Example 1 (Safety of low molecular weight LPS in crustaceans)

Kuruma prawns having an average weight of 20 g were divided into 5 groups of [each] each 20 prawns. The low molecular weight LPS of the present invention was intramascularly administered to the third abdominal segment of prawns in Groups 1 and 2 at a dose of 50 mg and 100 mg, respectively per kilogram of the prawn's weight. On the other hand, a conventional high molecular weight LPS (derived from E. coli, E. coli 0111 manufactured by DIFCO CO.) was intramascularly administered to the third abdominal segment of prawns in Groups 3 and 4 at a dose of 10 mg and 20 mg, respectively per kilogram of the prawn's weight. Group 5 received a physiological saline

free of LPS. The life or death of prawns up to 120 hours after administration was checked to determine mortality. The results are shown in Table 1.

Table 1

Group	number of perish / number tested	mortality (%)
Group 1 low MW LPS 50 mg/kg	0/20	0
Group 2 low MW LPS 100 mg/kg	0/20	0
Group 3 high MW LPS 10 mg/kg	13/20	65
Group 4 high MW LPS 20 mg/kg	20/20	100
Group 5 physiological saline	0/20	0

As apparent from Table 1, mortality of prawns in the groups receiving 10 mg or 20 mg of high molecular weight LPS was 65 or 100%, respectively, whereas no prawns died in the groups receiving 50 mg and 100 mg of low molecular weight LPS. It is clear from the above data that low molecular weight LPSs are significantly safe for prawns as compared with conventional high molecular weight LPSs.

Example 2 (Safety of low molecular weight LPS in fishes)

Black carps having an average weight of 85 g were divided into 3 groups of each 40 carps. The low molecular weight LPS of the present invention was intramascularly administered to the dorsal region of black carps in Group 1 at a dose of 100 mg per kilogram of the carp's weight. On the other hand, a conventional high molecular weight LPS (trade name E. coli 0111 manufactured by DIFCO CO.) was intramascularly administered to

the dorsal region of black carp in Group 2 at a dose of 20 mg per kilogram of the carp's weight. Group 3 received a physiological saline free of LPS. The life or death of black carp up to 120 hours after administration was checked to determine a mortality. The results are shown in Table 2.

Table 2

Group	number of perish / number tested	mortality (%)
Group 1 low MW LPS 100 mg/kg	0/40	0
Group 2 high MW LPS 20 mg/kg	34/40	85
Group 3 physiological saline	0/40	0

As apparent from Table 2, a mortality of black carp was 85% in the group receiving 20 mg of high molecular weight LPS, whereas no black carp died in the group receiving 100 mg of low molecular weight LPS. It is clear from the above data that the low molecular weight LPS of the present invention is significantly safe for fishes as compared with conventional high molecular weight LPS.

Example 3 (Activity of activating phagocytosis in hemocytes of crustaceans)

Kuruma prawns having an average weight of 20 g were divided into 6 groups of each 20 prawns. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with feeds at a daily dose of 20, 40 and 100 μg, respectively per kilogram of prawn's weight. On the other hand, Group 4 received a high molecular weight LPS as admixed with a

feed at a daily dose of 100 μg, and Group 5 received the same at a daily dose of 1000 μg, per kilogram of each prawn's weight. The feeds were given for 7 days. Group 6 was given a feed free of LPS. On days 0, day 1, day 5 and day 7 after supply of the feeds, the blood was collected from the thorax recess of prawns using a syringe holding a K-199 culture medium containing L-cystein as an anticoagulant. Hemocyte cells were obtained by centrifugation. The obtained cells (1×10^5 cells per microliter of the suspension) were mixed with 1×10^8 latex beads (1.986 μm in diameter), and were reacted at 25°C for 30 minutes. After fixing the reaction mixture with glutaraldehyde, it was air-dried. Then the mixture was subjected to giemsa stained staining and was fixed to a slide glass with Eukitt. The same procedure was repeated to obtain five samples per prawn. The hemocyte cells (200 cells per sample) were observed at random under an epi-fluorescent microscope to determine the phagocytic index after LPS stimulation phagocytosis ratio of latex beads in hemocyte and the number of latex beads phagocytized into one cell of hemocyte. Then the phagocytosis phagocytic index was calculated by the following equation:

Phagocytosis ratio=[number of hemocyte cells taking beads/total number of hemocyte cells observed] × 100.

Average number of beads taken phagocytosed by hemocyte cells = number of beads taken phagocytosed by hemocyte cells/number of hemocyte cells taking with phagocytosed beads.

Phagocytosis index=[number of hemocyte cells taking beads/total number of hemocyte cells observed] × [number of beads taken by hemocyte cells/total number of hemocyte cells observed] × 100.

Test results: The biophylaxis of crustaceans involves a cell

EUKITT (mounting medium, O. Kindler GmbH & Co. Freiburg, Germany)

phagocytosis ratio × average number of beads phagocytosed by hemocyte cells × 100

factor and a liquid factor. The phagocytosis of foreign particles in hemocytes is deeply concerned with the former. When the phagocytosis of foreign particles in prawn hemocytes is assessed, it is clarified whether the defensive mechanism of prawns is activated. [Yukinori TAKAHASHI et al, Research of Fish Diseases, 30 (2), pp.141 to 150, (1995)]. In view of said theory, the phagocytosis index was determined on days 0, day 1, day 5 and day 7 after supply of feeds for the groups receiving high molecular weight LPSs and the groups receiving the low molecular weight LPSs. The results were tabulated in Table 3.

Table 3

Group	Phagocytosis index of hemocyte 0	1 day	
Group 1 low MW LPS 20 μg/kg	0.9±0.18	2.1±0.61	*2
Group 2 low MW LPS 40μg/kg	0.9±0.18	3.3±1.16	*2
Group 3 low MW LPS 100μg/kg	0.9±0.18	3.8±1.00	*2
Group 4 high MW LPS 100μg/kg	0.9±0.18	0.7±0.31	
Group 5 high MW LPS 1000μg/kg	0.9±0.18	1.1±0.63	
Group 6 feed free of LPS	0.9±0.18	0.5±0.24	

Table 3 (continued)

Group	Phagocytosis index of hemocyte 5 days		7 days	
Group 1 low MW LPS 20 μg/kg	3.2±0.71	*2	8.4±1.37	*2
Group 2 low MW LPS 40μg/kg	4.5±0.75	*2	3.7±1.02	*2
Group 3 low MW LPS 100μg/kg	3.1±0.94	*2	2.8±0.70	*1
Group 4 high MW LPS 100μg/kg	0.7±0.82		1.2±0.44	
Group 5 high MW LPS 1000μg/kg	2.1±0.58	*1	2.9±0.68	*1
Group 6 feed free of LPS	0.7±0.5		1.1±0.56	

*1: Significant difference between this group and Group 6 (P<0.05)

*2: Significant difference between this group and Group 6 (P<0.01)

As apparent from Table 3, the groups receiving the low molecular weight LPSs (present invention) showed a higher phagocytosis index in hemocytes of prawns than Group 6, and a significant difference in this index from Group 6 (P<0.01, P<0.05). The group receiving 100 μg of conventional high molecular weight LPS was unable to increase the phagocytosis index in hemocytes of prawns after 1, 5 and 7 days. However, the group receiving 1000 μg of conventional high molecular weight LPS showed a significantly higher phagocytosis index in hemocytes of prawns (P<0.05) than Group 6 after 5 and 7 days. The above data show that the low molecular weight LPSs of the present invention can activate the defensive mechanism such as phagocytosis in hemocytes of prawns, even when used in an

extremely smaller amount than the high molecular weight LPSs.

Example 4 (Activity of activating phenol oxidase in hemocytes of crustaceans)

Kuruma prawns having an average weight of 20 g were divided into 6 groups of each 20 prawns. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with feeds at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of prawn's weight. Group 4 received a high molecular weight LPS as admixed with a feed at a daily dose of 100 μg, and Group 5 received the same as admixed with a feed at a daily dose of 1000 μg, per kilogram of prawn's weight. The supply of the feeds continued for 7 days. Group 6 received a LPS-free feed. The blood was collected from the thorax recess of prawns using a syringe holding a KHE culture medium having EDTA on days 0, day 1, day 5 and day 7 after supply of feeds. The collected blood was centrifuged to obtain hemocyte cells. The obtained cells were suspended in a Ca-Mg Hepes culture medium to a concentration of 1 X 10^6 cells/ml. The cells were crushed by freeze resolution and supersonic waves. The supernatant was separated off by centrifugation and was filtered with a membrane filter. The obtained filtrate (900 μl) was mixed with 100 μl of L-DOPA solution as a substrate solution. Thereafter the mixture was reacted at a temperature of 60°C for 60 minutes. Then the absorbance at 490 nm was measured by a spectrophotometer to assess a phenol oxidase activity (PO activity).

Test results: The biophylaxis of crustaceans involves a cell

factor and a liquid factor. The PO activity in hemocytes is deeply concerned with the latter. Thus, it is clarified by assessment of PO activity whether the defensive mechanism of prawns is activated. The PO activity of prawns was determined on days 0, day 1, day 5 and day 7 after supply of feeds for the groups receiving the low molecular weight LPSs (present invention) and the groups receiving high molecular weight LPSs. The results were tabulated in Table 4.

Table 4

Group	PO activity (absorbance·490nm) 0	1 day	5 days	7 days
Group 1 low MW LPS 20 μg/kg	0.092	0.105	0.199 *1	0.405 *2
Group 2 low MW LPS 40μg/kg	0.092	0.115	0.201 *1	0.325 *2
Group 3 low MW LPS 100μg/kg	0.092	0.166 *1	0.170 *1	0.292 *2
Group 4 high MW LPS 100μg/kg	0.092	0.093	0.124	0.138
Group 5 high MW LPS 1000μg/kg	0.092	0.104	0.197 *1	0.230 *1
Group 6 feed free of LPS	0.092	0.093	0.136	0.123

*1: significant difference between this group and Group 6 (P<0.05)

*2: significant difference between this group and Group 6 (P<0.01)

As apparent from Table 4, the groups receiving the low molecular weight LPSs (present invention) indicated a higher PO

activity than Group 6 and a significant difference in this activity from Group 6 ($P<0.01$, $P<0.05$). The group receiving 100 μg of conventional high molecular weight LPS did not increased in PO activity in hemocytes of prawns up to 7 days. The group receiving 1000 μg of conventional high molecular weight LPS showed a significantly higher PO activity in hemocytes of prawns ($P<0.05$) than Group 6 after 5 and 7 days. The above data show that the low molecular weight LPSs of the present invention can activate the defensive mechanism such as PO activity in hemocytes of prawns even when used in an extremely smaller amount than the high molecular weight LPSs.

Example 5 (Effect of preventing acute viremia in kuruma prawns)

Kuruma prawns having an average weight of 14 g were divided into 5 groups of each 20 prawns. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with feeds at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of prawn's weight. Group 4 received a high molecular weight LPS as admixed with a feed at a daily dose of 1000 μg, per kilogram of prawn's weight. Group 5 received peptidoglycan (PG) derived from Bifidobacterium thermophilum (Patent No.2547371) as admixed with a feed at a daily dose of 0.2mg (200 μg), per kilogram of prawn's weight. Group 6 received β-1,3-glucan (1,3-G) derived from Schizophyllum commune (JP-B-6-65649) as admixed with a feed at a daily dose of 50mg (50000 μg), per kilogram of prawn's weight. The supply of feeds continued for 18 days. Group 7 (control group) was given an LPS-free feed.

On day 8 after the start of supply of LPS, infection test was conducted using PRDV (penaeid rod-shaped DNA virus) as a pathogen inducing acute viremia in prawns. Carapaces were removed from the cephalothorax of three prawns which died of acute viremia. The intestine of prawns was crushed and homogenized in 40 ml of sterile seawater. The supernatant (10 ml) was separated off by centrifugation (10,000 × g, 10 minutes, 4°C) and added to 20 liters of seawater. On day 8 after the start of supply of LPS, prawns were infected with acute viremia by immersion in the supernatant for 2 hours. The life or death of prawns was observed for 10 days after infection. The dead prawns were pathologically tested and examined by PCR (polymerase chain reaction) method to confirm whether the prawns died of infection with PRDV.

Test results: Tables 5 and 6 show the total number of dead prawns and a mortality after infection with PRDV in the groups receiving low molecular weight LPSs of the present invention, the group receiving a high molecular weight LPS and the group receiving a LPS-free feed.

Table 5

Group	Days after infection 1	2	3	4	5
Group 1 low MW LPS 20 μg/kg	0	0	0	2*	3
Group 2 low MW LPS 40μg/kg	0	0	3	4	4
Group 3 low MW LPS 100μg/kg	1	1	3	3	4
Group 4 high MW LPS 1000μg/kg	1	1	6	6	6
Group 5 PG 0.2mg/kg	0	0	2	5	5
Group 6 1,3-G 50mg/kg	0	3	5	7	10
Group 7 feed free of LPS	2	4	13	14	15

* The number indicates the total number of dead prawns. (Other numbers show the same.)

Table 6

Group	Days after infection 6	7	8	9	10	Mortality
Group 1 low MW LPS 20 μg/kg	3	3	4	4	4	20 ***
Group 2 low MW LPS 40μg/kg	6	6	6	7	7	35 ***
Group 3 low MW LPS 100μg/kg	5	6	8	8	8	40 ***
Group 4 high MW LPS 1000μg/kg	9	9	10	11	11	55 **
Group 5 PG 0.2mg/kg	7	8	8	8	10	50 **
Group 6 1,3-G 50mg/kg	10	11	11	12	12	60 **
Group 7 feed free of LPS	18	18	19	20	20	100

** : significant difference between this group and Group 7 (P<0.05)

*** : significant difference between this group and Group 7 (P<0.01)

All (100%) of prawns died in the control group receiving an LPS-free feed up to 9 days after infection with PRDV. On the other hand, 20%, 35% and 40% of prawns died in the groups receiving 20, 40 and 100 μg, respectively, of low molecular weight LPS (present invention). In other words, a low mortality rate was observed for these groups, and a significant difference (P<0.01) exists between these groups and the control group. In contrast, 55% of prawns died in the group receiving 1000 μg of high molecular weight LPS, which means that more prawns died in this group than the groups receiving the low molecular weight LPSs. The above data demonstrate that the low molecular weight LPSs of the present invention can prevent viral infection of prawns and that the low molecular weight LPSs are more efficacious than conventional high molecular weight LPSs.

Example 6 (Activation of immune function in fishes)

Yellowtails weighing 230 g on an average were divided into 6 groups of each 20 yellowtails. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with moist pellets at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of each yellowtail's weight. Group 4 received a high molecular weight LPS as admixed with moist pellets at a daily dose of 100 μg, and Group 5 received a high molecular weight LPS as admixed with moist pellets at a daily

dose of 1000 μg, per kilogram of yellowtail's weight. The feeds were given for 7 days. Group 6 received LPS-free moist pellets. On days 0, day 1, day 5 and day 7 after supply of feeds, a head kidney was excised from 5 yellowtails. Then Hemocyte cells were separated in a plastic petri dish holding a 0.25% NaCl containng RPMI-1640-HAH culture medium. The cells were passed through a cell filter to give a cell suspension. The suspension was placed over a discontinuous Percoll density gradient. Thereafter a leukocyte layer was formed by centrifugation (1600 rpm., at 4°C for 20 minutes).

The leukocyte layer was collected and was subjected to centrifugal washing after which the cells were suspended in a 10% FBS (fetal bovine serum)-containing 0.25% NaCl-including RPMI-1640-H culture medium. The number of leukocyte cells in the suspension was adjusted to 1 X 10^6 cells/ml. The leukocyte suspension (500 μl) and 500 μl of a suspension (1 × 10^8 cells/ml) of yeast opsonized with serum of yellowtail were placed into a silicone-treated glass test tube and were incubated at 25°C for 60 minutes with stirring every 10 minutes. After incubation, 5 smears per yellowtail were produced, subjected to Wright's staining and enclosed with Eukitt. The hemocyte cells (200 cells per smear) were observed at random under an optical microscope. Then the number of yeast cells phagocytized into leukocytes was counted. The phagocytosis index was given by the same equation as in Example 3. The results are shown in Tables 7 and 8.

Table 7

Group	Phagocytosis index of leukocyte 0	1 day	
Group 1 low MW LPS 20 μg/kg	7.3±2.30	12.7±2.65	*1
Group 2 low MW LPS 40μg/kg	7.3±2.30	17.9±3.99	*2
Group 3 low MW LPS 100μg/kg	7.3±2.30	18.6±4.12	*2
Group 4 high MW LPS 100μg/kg	7.3±2.30	6.3±2.24	
Group 5 high MW LPS 1000μg/kg	7.3±2.30	8.2±2.18	
Group 6 feed free of LPS	7.3±2.30	6.6±1.19	

*1: significant difference between this group and Group 6 (P<0.05)

*2: significant difference between this group and Group 6 (P<0.01)

Table 8

Group	Phagocytosis index of leukocyte 5 days		7 days	
Group 1 low MW LPS 20 μg/kg	39.2±2.54	*2	52.7±4.08	*2
Group 2 low MW LPS 40μg/kg	37.4±4.28	*2	37.0±3.11	*2
Group 3 low MW LPS 100μg/kg	42.6±5.35	*2	36.5±4.32	*1
Group 4 low MW LPS 100μg/kg	11.2±3.05		10.6±2.96	
Group 5 low MW LPS 1000μg/kg	22.7±3.16	*1	31.8±3.52	*1
Group 6 feed free of LPS	9.0±2.04		7.7±1.73	

*1: significant difference between this group and Group 6 (P<0.05)

**2: significant difference between this group and Group 6

(P<0.01)

As apparent from Tables 7 and 8, any groups of yellowtails receiving the low molecular weight LPSs (present invention) indicated a higher phagocytosis index in leukocytes of yellowtails than Group 6, and a significant difference (P<0.01, P<0.05) in this index from Group 6. However, the group receiving 100 μg of conventional high molecular weight LPS did not increase the phagocytosis index in leukocytes of yellowtails after 7 days. The group receiving 1000 μg of conventional high molecular weight LPS showed a significantly higher phagocytosis index (P<0.01) in leukocytes of yellowtails than Group 6 after 5 days. The above data show that the low molecular weight LPSs of the present invention can activate the immune system of fishes such as phagocytosis in leukocytes in an extremely smaller amount than conventional high molecular weight LPSs.

Example 7 (Effect of preventing enterococcus disease in yellowtails)

Yellowtails weighing 63 g on an average were divided into 5 groups of each 30 yellowtails. Groups 1, 2 and 3 received the low molecular weight LPSs of the present invention as admixed with moist pellets at a daily dose of 20, 40 and 100 μg, respectively, per kilogram of shrimp's weight. Group 4 received a high molecular weight LPS as admixed with moist pellets at a daily dose of 1000 μg per kilogram of yellowtail's weight. Group 5 (control) received LPS-free moist pellets. On day 7 after supply of feeds, the yellowtails were intraabdominally inoculated with Enterococcus Seriolicida as a

pathogen causing enterococcus disease of yellowtail in an amount of 4.0 X 10^6 cells per yellowtail. The mortality rate 15 days after inoculation was determined. The results are shown in Tables 9 and 10.

Table 9

Group	Days after infection 1	2	3	4	5	6	7	8	9
Group 1 low MW LPS 20 μg/kg	0	0	0	0	0	0	0	0	1*
Group 2 low MW LPS 40μg/kg	0	0	0	1	1	2	2	4	4
Group 3 low MW LPS 100μg/kg	0	0	0	0	0	1	3	3	5
Group 4 high MW LPS 1000μg/kg	0	0	0	1	1	1	3	3	3
Group 5 feed free of LPS	0	0	1	2	7	7	10	12	16

* The number indicates the total number of dead yellowtails. (Other numbers show the same.)

Table 10

Group	Days after infection 10	11	12	13	14	15	Mortality (%)
Group 1 low MW LPS 20 μg/kg	3	3	3	3	4	4	13.3 ***
Group 2 low MW LPS 40μg/kg	7	8	8	8	8	8	26.7 **
Group 3 low MW LPS 100μg/kg	5	5	5	7	7	7	23.3 **
Group 4 high MW LPS 1000μg/kg	5	9	10	10	11	11	36.7 **
Group 5 feed free of LPS	16	16	17	22	22	22	73.3

** : significant difference between this group and Group 5 ($P<0.05$)

***: significant difference between this group and Group 5 ($P<0.01$)

On 15th days after inoculation of E. Seriolicida, 73.3% of yellowtails died in the control group receiving LPS-free feed. In contrast, a low mortality is indicated by the groups receiving the low molecular weight LPSs of the present invention, i.e. 13.3% from the group receiving 20 μg, 26.7% from the group receiving 40 μg and 23.3% from the group receiving 100 μg. In other words, there is a significant difference ($P<0.05$) in mortality between these groups and the control group. On the other hand, a mortality of 36.7% resulted from the group receiving 1000 μg of high molecular weight LPS. This group showed a higher mortality than the groups receiving low molecular weight LPSs. The above results show that the low molecular weight LPSs of the present invention can protect fishes against viral infection and are more efficacious than conventional high molecular weight LPSs.

INDUSTRIAL APPLICABILITY

According to the present invention, there is provided a safe feedstuff additive for growing crustaceans and fishes, the feedstuff additive being capable of preventing infectious diseases by properly activating their intrinsic immune function even when used in a small amounts being capable of preventing the perish of crustaceans and fishes, and being free from the problems of public health hazards such as the feedstuff additive remaining in crustaceans and fishes.